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# Molecular Cloning of a New Interferon-induced PML Nuclear Body-associated Protein\*

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**Transcriptional induction of genes is an essential part of the cellular response to interferons. We have established a cDNA library from human lymphoblastoid Daudi cells treated for 16 h with human  $\alpha/\beta$ -interferon (IFN) and made use of differential screening to search for as yet unidentified IFN-regulated genes. In the course of this study, we have isolated a human cDNA that codes for a 20-kDa protein sharing striking homology with the product of the *Xenopus laevis* XPMC2 gene. This new gene is induced by both type I and II IFNs in various cell lines and will be referred to as ISG20 for interferon-stimulated gene product of 20 kDa. Confocal immunofluorescence analysis of the subcellular localization of ISG20 protein reveals that it is closely associated with PML and SP100 gene products within the large nuclear matrix-associated multiprotein complexes termed the PML nuclear bodies.**

The interferons (IFNs)<sup>1</sup> are a family of secreted multifunctional proteins that exert a broad spectrum of biological activities. First characterized for their potent antiviral properties, it has now been established that they are involved in a number of regulatory functions such as control of cell proliferation, differentiation, and regulation of the immune system (1, 2). Binding of both type I IFN (IFN- $\alpha/\beta$ ) and type II IFN (IFN- $\gamma$ ) to different cell-surface receptors (3, 4) activates transduction pathways via tyrosine phosphorylation of latent cytoplasmic transcription factors termed STAT factors (for signal transducer and activator of transcription) (5–11). The STAT factors are assembled to form the specific transcription complexes ISGF3 (for interferon-stimulated gene factor 3) for IFN- $\alpha/\beta$  and GAF (for IFN- $\gamma$  activation factor) for IFN- $\gamma$ . These transcription factors act at different *cis*-acting DNA elements termed the IFN-stimulated responsive element for ISGF3 and the IFN- $\gamma$  activation site for GAF and are located in the promoter region of IFN-induced genes (12–18).

The diverse biological actions of IFNs are thought to be

mediated by the products of specific but usually overlapping sets of cellular genes induced in the target cells. More recently, some interferon-induced proteins have been located within discrete nuclear structures termed nuclear bodies (19–22). Previously defined by electron microscopy as dense 0.3–0.5-nm diameter spherical particles, the nuclear bodies, so-called PML (for promyelocytic leukemia protein) nuclear bodies (PML NBs), appear characteristic of large multiprotein complexes associated with the nuclear matrix (23–28). These structures are distinct from other well described subnuclear domains such as the nucleolus, the interchromatin granules, the perichromatin fibrils, and the coiled bodies (for review, see Ref. 29). Among the PML NB-associated proteins, PML is the best documented. PML was originally discovered as a fusion protein with retinoic acid receptor- $\alpha$  in malignant hematopoietic cells with a t(15:17) translocation characteristic for patients with acute promyelocytic leukemia (reviewed in Refs. 30–32). A tumor cell growth suppressor function and a role in human oncogenesis were reported for PML (33–36). In addition to PML, the nuclear bodies include at least two other IFN-induced proteins, NDP52 (for nuclear dot protein 52) (19) and the SP100 protein, originally identified as an autoantigen in patients with primary biliary cirrhosis (20, 24). It is interesting to note that the number and morphology of PML NBs are variable, particularly throughout the cell cycle and in some pathological contexts. In acute promyelocytic leukemia, PML NBs are disrupted into a microparticulate pattern as a consequence of the expression of the PML-retinoic acid receptor- $\alpha$  oncoprotein. Retinoic acid treatment triggers a reorganization of the nucleus to generate normal appearing PML NBs, which in turn is linked to differentiation of acute promyelocytic leukemia cells (26–28).

Although the function of PML NBs is still unknown, some observations suggest that they may represent preferential targets for viral infection and thus could play a role in the mechanism of antiviral action of IFNs. In particular, after adenovirus infection, the viral E4-ORF3 protein is targeted to PML NBs and causes their reorganization from spherical to fibrous structures (37, 38). The human T-cell leukemia virus type 1 Tax oncoprotein induces a diffuse cytoplasmic redistribution of the Int-6 protein, which normally colocalizes with PML in the absence of Tax expression (39). This delocalization appears to be specific for Int-6 because Tax does not alter the global speckled staining pattern of PML. The herpes simplex virus type 1 immediate-early protein Vmw110 (ICPO), which is implicated in the control of reactivation of latent herpes simplex virus type 1, transiently colocalizes after viral infection to PML NBs and subsequently disrupts these structures (40, 41). In the same way, PML NBs are reorganized after human cytomegalovirus infection (42, 43). The Epstein-Barr virus-encoded nuclear antigen EBNA-5 (44), the adenovirus E1A protein, and the SV40 large T antigen (37), other viral members of the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X89773.

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<sup>1</sup> The abbreviations used are: IFNs, interferons; HuIFN, human interferon; PML NBs, PML nuclear bodies; HA, hemagglutinin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ORF, open reading frame.

oncprotein family, are also found in close association with PML NBs. These observations, coupled with the fact that the PML, SP100, and NDP52 proteins are all induced by IFN (19–22), have suggested that PML NBs may play a role in the viral infection process. The elucidation of the actual role of PML NBs in the cell and their implication in the mechanism of action of IFN will be through the identification of new PML NB-associated proteins and the characterization of their behavior under physiological stimuli.

We have therefore established a cDNA library from IFN-treated Daudi cells and made use of differential screening to search for as yet unidentified IFN-regulated genes (45). In the course of this study, we have isolated a human cDNA encoding a novel protein that shares strong similarity with the nuclear *Xenopus* protein XPMC2 (46). This new gene will be referred to as *ISG20* for interferon-stimulated gene product of 20 kDa. Using laser confocal immunofluorescence analysis, we demonstrate that *ISG20* protein is closely associated with PML and SP100 in the newly described nuclear structures termed PML NBs.

#### MATERIALS AND METHODS

**Cell Cultures and Antibodies**—Human lymphoblastoid Daudi cells were grown in suspension in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum. Hamster fibroblast (CCL39), SV40-transformed monkey kidney epithelial (COS-7m6), human hepatocarcinoma (CCL13), and human HeLa cells were grown in monolayer cultures in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum. For IFN induction, exponentially growing cells were exposed for 16 h to 500 IU/ml human lymphoblastoid IFN (HuIFN- $\alpha/\beta$ ; obtained from Hayashibara Biochemical Laboratories Inc.) or 500 units/ml IFN- $\gamma$  (a gift of Roussel-UCLAF, Paris, France). For the generation of anti-PML and anti-SP100 antibodies, a PML cDNA *Nco*I-*Sma*I fragment (positions 80–1421 in the cDNA sequence) and the full-length coding region of SP100 cDNA were inserted into a glutathione *S*-transferase gene fusion vector to generate GST-PML and GST-SP100 hybrid proteins. Recombinant proteins produced in *Escherichia coli* were purified using glutathione-Sepharose 4B columns and were used for rabbit immunizations.

**RNA Purification and Northern Blot Analysis**—For RNA purification, the cells were pelleted and washed in phosphate-buffer saline, and total mRNAs were isolated by the guanidine thiocyanate method as described previously (47). RNAs were fractionated by electrophoresis on a 10% (v/v) formaldehyde-containing 1.2% (w/v) agarose gel and transferred to nylon membranes (Hybond-N, Amersham Corp.). The multiple-tissue Northern blot membrane (CLONTECH) was a gift of Dr. P. Fort. Prehybridizations were performed at 42 °C for 12 h in a mixture containing 50% (v/v) formamide, 0.75 M NaCl, 50 mM sodium phosphate, pH 7, 1 mM EDTA, 0.2% (w/v) SDS, 5 × Denhardt's solution, 10% (w/v) dextran sulfate, and 100 mg/ml denatured salmon sperm DNA. An additional 12-h hybridization was performed in the presence of 10<sup>6</sup> cpm/ml <sup>32</sup>P-labeled random-primed cDNA probe. Stringent washings were then conducted at 65 °C in 0.1 × SSC (0.15 M NaCl and 0.015 M sodium citrate) before autoradiography.

**Determination of Transcriptional Activity**—For run-on experiments, the cells were washed twice with phosphate-buffered saline and lysed with a Dounce homogenizer in 10 volumes of 0.25 M sucrose containing 10 mM Tris-HCl, pH 8.3, 10 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 0.5% (v/v) Nonidet P-40. Nuclei were purified through a sucrose cushion as described previously (48).

**Construction of cDNA Library and Isolation of cDNA Clones**—Poly(A<sup>+</sup>) RNAs were isolated from total mRNAs using the Dynabeads biomagnetic separation system (Biosys S. A.), and the cDNA library was constructed in the  $\lambda$ ZAP cDNA synthesis system (Stratagene). The library was plated at low density to obtain individual plaques and transferred to nylon membranes (Hybond-N). A single round of screening was performed by successive hybridization of a single filter using <sup>32</sup>P-labeled cDNA probes (2 × 10<sup>6</sup> cpm/ml) obtained from poly(A<sup>+</sup>) RNAs of untreated or IFN-treated Daudi cells. Prehybridization, hybridization, and washing of the filter were performed as described for Northern blot analysis. Clones exhibiting a variation in signal intensity were isolated, and the pBluescript phagemid vectors containing inserts were excised using the ExAssit-SORL system (Stratagene). Phagemid DNAs were then extracted and used to probe Northern blot membranes.

**Sequence Determination and Characterization of cDNA Clones**—Plasmid DNAs of individual clones were prepared, and their sequences were determined by the Sanger dideoxy sequencing method (T7 sequencing kit, Pharmacia Biotech Inc.). The complete sequence of *ISG20* cDNA was obtained on both strands by overlapping sequenced fragments of the original clone after subcloning in the pBluescript II KS vector. Searches for sequence homologies in the EMBL and GenBank™ Data Banks as well as sequence analyses were performed by using the BISSANCE facilities (49).

**Transfection Assays and Immunofluorescence**—CCL13, CCL39, and COS cells were grown in Dulbecco's modified Eagle's medium supplemented with glutamine, antibiotics, and 10% fetal calf serum (Life Technologies, Inc.). Transfections were performed by the calcium phosphate precipitation method for CCL13 and by the LipofectAMINE procedure (as outlined by Life Technologies, Inc.) for CCL39 and COS cells. Cells were grown as monolayers on glass coverslips in 35-mm Petri dishes containing 2 ml of culture medium and transfected with 2.5  $\mu$ g of expression vector DNA and 2.5  $\mu$ g of carrier pSG5 plasmid. 24 h after transfection, the medium was replaced by fresh culture medium, and the cells were further incubated for 24 h. Immunofluorescence was performed as previously described (26) with anti-PML or anti-SP100 polyclonal antibodies (diluted 1:200) and with the 12CA5 anti-HA monoclonal antibody (15 ng/ $\mu$ l). Slides were viewed using a Leica confocal microscope. Image files were processed with the Adobe Photoshop program.

#### RESULTS

**Construction and Screening of cDNA Library from HuIFN- $\alpha/\beta$ -treated Daudi Cells**—Total RNAs were extracted from human lymphoblastoid Daudi cells treated for 16 h with 500 IU of HuIFN- $\alpha/\beta$ . An oriented cDNA library was constructed using the Stratagene  $\lambda$ ZAP cDNA synthesis kit. 5000 primary recombinant clones were screened successively with single-stranded <sup>32</sup>P-labeled cDNA derived from exponentially growing untreated cells and with cDNA from IFN-treated cells. Spots exhibiting a variation in signal intensity were selected, and pBluescript phagemid vectors containing inserts were excised using the Stratagene ExAssit-SORL system. DNAs were prepared and used to probe a Northern blot containing total RNA extracted from Daudi cells treated for various times with HuIFN- $\alpha/\beta$ . Clones exhibiting differential expression upon Northern blot analysis by comparison with an invariant glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (50) probe were selected. Complete sequence examination of positive clones revealed that one that contains a 694-nucleotide insert encoded a new IFN-induced mRNA, which will be referred to as *ISG20*.

**Analysis and Specificity of Expression of New IFN-induced RNA**—The kinetics of expression of the RNA hybridizing to the *ISG20* cDNA probe was analyzed by probing a Northern blot of total RNAs isolated from Daudi cells treated for various times with HuIFN- $\alpha/\beta$ . As shown in Fig. 1A, the *ISG20* mRNA accumulated rapidly after the onset of IFN treatment. An 8-fold increase in its steady-state level was reached after 16 h of exposure to IFN. Hybridization to a *GAPDH* probe used as an invariant control confirmed that each lane of the blot contained an equal amount of total RNA (Fig. 1A). *ISG20* participates in the primary response of IFN action since this induction was not dependent on continuous protein (data not shown).

The specificity of induction of the *ISG20* mRNA in response to treatment with the various types of IFN was then analyzed. As Daudi cells failed to respond to HuIFN- $\gamma$  due to the lack of functional receptors, HeLa cells were treated with 500 units/ml HuIFN- $\alpha/\beta$  or HuIFN- $\gamma$ , and total RNAs were extracted and analyzed as described above. As shown in Fig. 1B, the kinetics of induction of *ISG20* mRNA was found to be similar with the two types of IFN, although HuIFN- $\alpha/\beta$  was found to be a stronger inducer.

To determine whether the modulation of *ISG20* occurs at the transcriptional level, nuclear run-on assays were conducted

with nuclei purified from Daudi cells treated or not with HuIFN- $\alpha/\beta$  as described previously (48). The labeled RNAs were hybridized with filters containing *ISG20*, GAPDH, and pBluescript II KS vector probes. GAPDH and the pBluescript II KS vector were used as invariant and negative controls, respectively. The data presented Fig. 1C clearly demonstrate that the increase in *ISG20* expression by IFN occurs at the transcriptional level, in keeping with the mechanism of induction of the majority of IFN-induced genes.

To determine the tissue specificity of *ISG20* expression, the 694-nucleotide insert was used to probe a set of RNAs isolated from several tissues (multiple-tissue Northern blot membrane from CLONTECH). As shown in Fig. 2, *ISG20* is strongly expressed, in the absence of exogenous IFN treatment, in peripheral blood leukocytes, in lymphoid tissues (such as spleen or thymus), and in colon and lung. Various basal levels were detected in other tissues. The same blots were stripped and rehybridized with the control  $\beta$ -actin provided by the manufacturer as an invariant control.

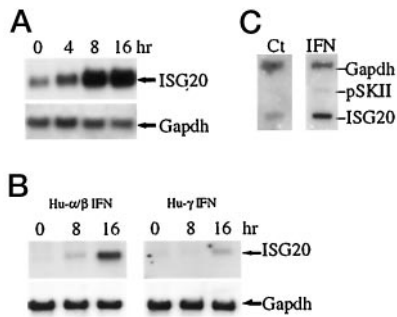
**Sequence Analysis of *ISG20* cDNA**—To investigate the function of *ISG20*, the nucleotide sequence of the 694-nucleotide *ISG20* cDNA fragment was determined. A single open reading frame (ORF) of 537 nucleotides was identified (Fig. 3). The *ISG20* ORF predicts a gene product (gpISG20) of 179 amino

acids with a relative molecular mass of 20.4 kDa. Analysis of the deduced amino acid sequence revealed a very basic protein with 7 lysine and 19 arginine residues (Lys + Arg = 14.5% of total residues) and a pI of 9.2. Using algorithms to predict the presence of  $\alpha$ -helices (51) and coiled-coil structures (52), region 78–107 of gpISG20 is strongly predicted to form a coiled-coil domain (Fig. 3). This structural domain is known to mediate protein-protein interactions (for review, see Ref. 53), suggesting that *ISG20* can act as a component of a multiprotein complex; this important point will be discussed later on the basis of the subcellular localization of *ISG20* protein. The coiled-coil region is bordered on both sides by potential phosphorylation sites. A tyrosine kinase phosphorylation site is located from amino acids 44 to 52, and a phosphorylation domain is located from amino acids 106 to 134, which contains several potential protein kinase C, casein kinase II, and cAMP-dependent protein kinase phosphorylation sites (Fig. 3).

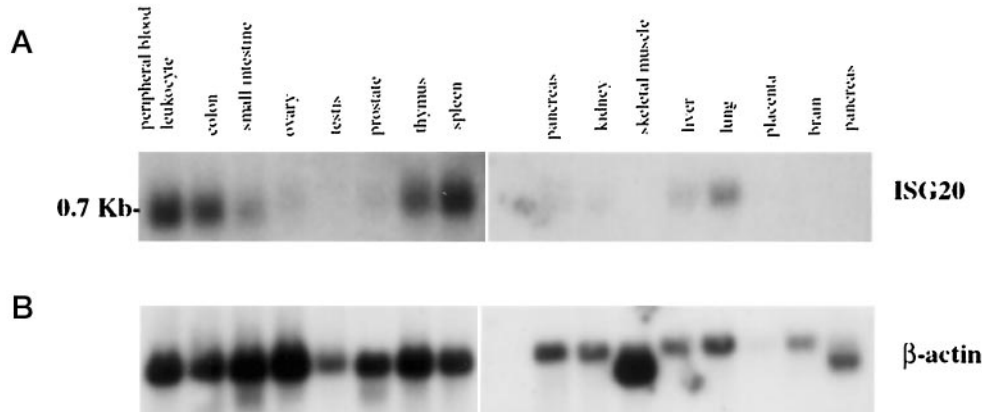
A nucleotide comparison by computer search did not reveal any significant homology between *ISG20* and the sequences referenced in data bases. However, a search for amino acid sequence homologies revealed that the complete *ISG20* protein shares amino acid cluster homologies with the *Xenopus laevis* XPMC2 gene product (46) and with a theoretical ORF present in the genome of *Saccharomyces cerevisiae* (GenBank™ accession number Z74822). Expression of XPMC2 cDNA has been shown to rescue in the fission yeast *Schizosaccharomyces pombe* several mitotic catastrophe mutants defective in both Wee1 and Mik1 kinases. These redundant kinases negatively regulate Cdc2 kinase by phosphorylating a conserved tyrosine residue. The XPMC2 gene product acts as a negative cell cycle regulator by competing with mitotic substrates for phosphorylation by Cdc2 kinase (46). The role and the regulation of the *S. cerevisiae* ORF remain unknown.

On the basis of the amino acid homology between *ISG20* and XPMC2, whose amino acid sequence alignment is presented in Fig. 4, it is tempting to speculate that *ISG20* may be one of the elements that participate in the negative regulation of cell division, which would be consistent with the anti-growth properties of IFNs as found in particular in Daudi cells. We evaluated this hypothesis by analysis of the cell cycle distribution of cells overexpressing *ISG20*. To this aim, COS-7m6 cells were transiently transfected with a vector expressing *ISG20*. Transfection was performed by the LipofectAMINE procedure. The cells were collected 72 h later and analyzed with a flow cytometer after propidium iodide staining. Under these experimental conditions, no significant alteration in the cell cycle distribution was observed (data not shown).

The recent release in the data bases of a human 5'-EST



**FIG. 1. Northern analysis of IFN-induced *ISG20* mRNA.** Total RNAs (20  $\mu$ g/lane) were separated on 1.2% formaldehyde-agarose gel, transferred to nylon membrane, and hybridized to a  $^{32}$ P-labeled *ISG20* cDNA probe. The same blot was reprobed with a GAPDH probe to ensure that equal amounts of RNA were loaded in each lane. **A**, time course of *ISG20* mRNA induction with HuIFN- $\alpha/\beta$ . Daudi cells were treated with HuIFN- $\alpha/\beta$  (500 units/ml) for the indicated times. **B**, specificity of *ISG20* mRNA induction by HuIFN- $\alpha/\beta$  and HuIFN- $\gamma$ . HeLa cells were treated with HuIFN- $\alpha/\beta$  (500 units/ml) or HuIFN- $\gamma$  (500 units/ml) for the indicated times. **C**, run-on assays of transcription elongation of the *ISG20* gene. Run-on assays were carried out with nuclei from Daudi cells treated (IFN) or not (control (Ct)) with HuIFN- $\alpha/\beta$  for 16 h. The labeled RNAs were hybridized with a filter containing the indicated probes. pSKII, pBluescript II KS vector.



**FIG. 2. Tissue specificity of *ISG20* expression.** **A**, a multiple-tissue Northern blot membrane (CLONTECH) was hybridized to a  $^{32}$ P-labeled *ISG20* cDNA probe. The tissue source of the mRNA sample in each lane is indicated at the top. The same blot was stripped and rehybridized with a  $\beta$ -actin cDNA probe as a control. Kb, kilobase.

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                                     M A G S R E V   7
CTGCAGAAATTCGGCACGAGCTCTGAGGGTCCCAAGGAAC ATG GCT GGG AGC CGT GAG GTG   61

V A M D C E M V G L G P H R E S G L   25
GTG GCC ATG GAC TGC GAG ATG GTG GGG CTG GGG CCC CAC CGG GAG AGT GGC CTG 115

A R C S L V N V H G A V L Y D K F I   43
GCT CGT TGC AGC CTC GTG AAC GTC CAC GGT GCT GTG CTG TAC GAC AAG TTC ATC 169

R P E G E I T D Y R T R V S G V T P   61
CGG CCT GAG GGA GAG ATC ACC GAT TAC AGA ACC CGG GTC AGC GGG GTC ACC CCT 223

Q H M V G A T P F A V A R L E I L V   79
CAG CAC ATG GTG GGG GCC ACA CCA TTT GCC GTG GCC AGG CTA GAG ATC CTG CAG 277

L L K G K L V V G H D L R H D F Q A   97
CTC CTG AAA GGC AAG CTG GTG GTG GGT CAT GAC CTG AAG CAC GAC TTC CAG GCA 331

L K E D M S G Y T I Y D T S T D R L   115
CTG AAA GAG GAC ATG AGC GGC TAC ACA ATC TAC GAC ACG TCC ACT GAC AGG CTG 385

L W R E A K L D H C R R V S L R V L   133
TTG TGG CGT GAG GCC AAG CTG GAC CAC TGC AGG CGT GTC TCC CTG CGG GTG CTG 439

S E R L L H K S I Q P L G H S S V E   151
AGT GAG CGC CTC CTG CAC AAG AGC ATC CAG CCG CTT GGA CAC AGC TCG GTG GAA 493

D A R A T M E L Y Q I S Q R I R A R   169
GAT GCG AGG GCA ACG ATG GAG CTC TAT CAA ATC TCC CAG AGA ATC CGA GCC CGC 547

R G L P R L A V S D   179
CGA GGG CTG CCC CGC CTG GCT GTG TCA GAC TGA AGC CCC ATC CAG CCC GTT CCG 601

CAG GGA CTA GAG GCT TTC GGC TTT TTG GGA CAG CAA CTA CCT TGC TTT TGG AAA 655

ATA CAT TTT TAA TAG TAA AGT GGC TCT ATA TTT TCT CTA   694

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FIG. 3. Nucleotide sequence and predicted amino acid sequence of ISG20 cDNA. The complete nucleotide sequence of ISG20 cDNA (bottom line) and the predicted amino acid sequence (top line) are shown. The nucleotides and amino acids are numbered to the right of the sequence. The predicted coiled-coil region is boxed. The two phosphorylation domains are underlined.

sequence (GenBank™ accession number R02224) with a high nucleotide identity to the XPMC2 cDNA clearly demonstrates that ISG20 is not the human homolog of XPMC2, but is a member of a new family of proteins. Surprisingly, human EST-like ISG20 corresponds to a short RNA and encodes a protein homologous only to the carboxylic half of the XPMC2 protein.

**Subcellular Localization of ISG20 Protein**—The ISG20 amino acid sequence does not enclose a canonic bipartite nuclear localization signal, but the presence of lysine- and arginine-rich domains and the small size of the protein suggest that gpISG20 might be targeted to the nucleus. To determine the subcellular localization of ISG20, CCL13, CCL39, and COS cells were transfected with a tagged ISG20 protein. A fusion cDNA between the open reading frame of ISG20 and the HA epitope peptide sequence of the influenza virus was cloned under the control of the cytomegalovirus promoter in the pJ7Ω vector (pJ7TagHA-ISG20). Cells were transfected by the calcium phosphate precipitation or LipofectAMINE method and then analyzed by immunofluorescence using the 12CA5 monoclonal antibody. The ectopically expressed ISG20 was predominantly nuclear and gave a speckled distribution pattern in all

cell types (Fig. 5A). This localization was not dependent on the level of tagged ISG20 expression since the same pattern was observed in COS cells, which express a high level of transfected cDNA as compared with CCL39 and CCL13 cells. However, in CCL13 cells, ISG20 was diffusely distributed throughout the nucleoplasm in ~30% of the positive cells. This percentage strongly suggests that the change in the intranuclear distribution of ISG20 might be dependent on the progression of the cell cycle. The nuclear dots containing ISG20 were dispersed throughout the nucleoplasm and were variable in size and number per nucleus (Fig. 5A). The speckled nuclear staining pattern of ISG20 is reminiscent of the labeling observed for other cellular proteins such as PML and SP100, which colocalize within large multiprotein complexes called PML NBs (23–28). To compare the intranuclear localization of ISG20 with that of endogenous PML, double staining immunofluorescence experiments were performed on the ISG20-transfected CCL13 cells using the 12CA5 monoclonal antibody and an anti-PML polyclonal antiserum (26). The punctuate pattern of overexpressed ISG20 is coincident with that of endogenous PML as shown in Fig. 5B. To examine whether ISG20 and PML exactly localized within the same nuclear structures, confocal immu-



FIG. 4. Alignment of amino acid sequences of ISG20 and XPMC2 proteins. The proteins are indicated on the left. The positions of the conserved amino acids are indicated by asterisks, and the conserved hydrophobic residues by periods.

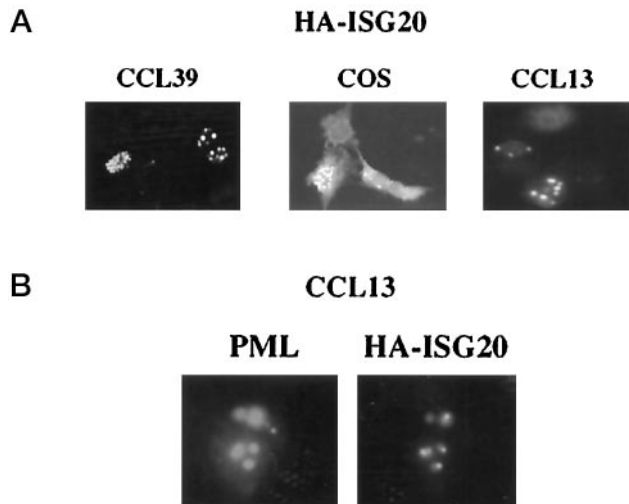


FIG. 5. Subcellular localization of ISG20 protein. A, nuclear localization by immunofluorescence of ectopic HA-ISG20 in transiently transfected CCL39, COS, and CCL13 cell lines; B, localization by double immunofluorescence labeling of ectopic hemagglutinin-ISG20 (right panel) and endogenous human PML (left panel) in CCL13 cells.

no fluorescence microscopic analysis was performed in the CCL13 cells. The localization of ectopically expressed ISG20 was compared with that of the two major endogenous components of NBs, the PML and SP100 proteins. As shown in Fig. 6, all of the nuclear dots containing ISG20 were coincident with the dots containing both PML and SP100 proteins as revealed by double labeling with the 12CA5 monoclonal antibody and PML (Fig. 6A) or SP100 (Fig. 6B) polyclonal antiserum. The ISG20 labeling pattern was closely associated rather than perfectly overlapping with the PML/SP100 immunofluorescence pattern. Moreover, there were additional PML or SP100 dots that did not contain detectable levels of ISG20 protein. It is not clear at present whether or not the level of ectopically expressed ISG20 protein is sufficient for occupying all the PML NB sites or whether this represents a preferential association of ISG20 with a subset of PML NBs.

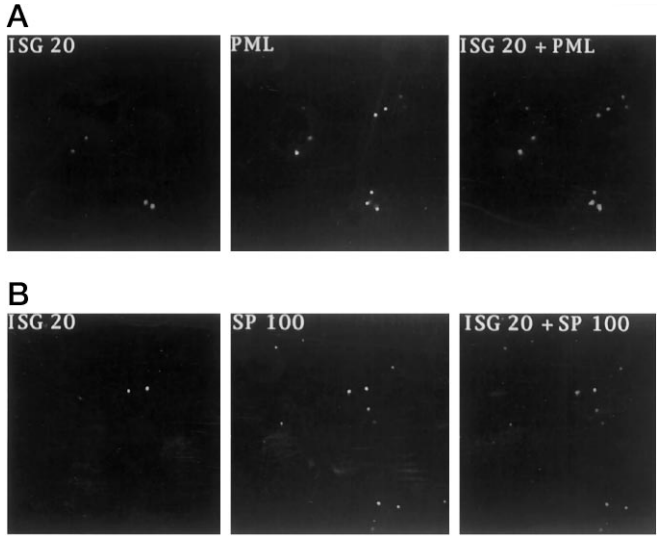


FIG. 6. Laser confocal immunofluorescence analysis of colocalization of ISG20 with PML and SP100 in CCL13 cell line. A, localization by double immunofluorescence labeling of ectopic HA-ISG20 (left panel) and endogenous human PML (center panel) in HA-ISG20-transfected CCL13 cells. The single confocal images were superimposed (right panel). B, localization by double immunofluorescence labeling of ectopic HA-ISG20 (left panel) and endogenous human SP100 (center panel) in HA-ISG20-transfected CCL13 cells. The single confocal images were superimposed (right panel).

DISCUSSION

Binding of IFNs to their specific cell-surface receptors triggers the rapid nuclear translocation of a complex formed by association between the various phosphorylated STAT proteins (see the Introduction). This mechanism results in the induction of specific sets of genes that mediate the various biological functions of IFNs. The proteins encoded by these genes exhibit cytoplasmic, nuclear, or cell-surface localization. Cytological analysis has revealed a complex functional organization within the nucleus. Some nuclear proteins localize within discrete and functionally distinct classes of nuclear domains. The two most documented are the nucleolus, in which genes are transcribed and ribosomal RNA is processed (54), and the presumptive

splice sites resulting from the association of small ribonucleoprotein particles with specific sites on the nuclear matrix (55–57). Recently, some IFN-induced proteins have been described firmly bound to the nuclear matrix, forming discrete nuclear structures termed NBs or PML NBs, distinct from the subnuclear domains previously described (19–28). In this report, we describe the isolation and characterization of a cDNA encoding a novel PML NB-associated protein designated ISG20, the expression of which is induced after HuIFN treatment.

Comparison of the amino acid sequence of ISG20 with the sequences of EMBL and GenBank™ Data Banks revealed significant homologies between ISG20 and the *X. laevis* XPMC2 gene product (46). Using a genetic complementation method, XPMC2 has been identified to functionally rescue a fission yeast mitotic catastrophe mutant defective in both Wee1 and Mik1 kinases (46). These homologies raise the possibility that ISG20 might act as a negative regulator of cell division induced by IFNs. However, ISG20 did not appear to affect cell growth in transient transfection experiments. Alignment of the amino acid sequences of ISG20 and XPMC2 showed that ISG20 is much shorter than XPMC2 and is homologous to the C-terminal half of XPMC2 protein. Interestingly, a truncated XPMC2 protein that retains only its C-terminal half is not able to rescue the mitotic catastrophe phenotype. According to the presence within ISG20 protein, of a presumptive structural coiled-coil domain that is supposed to mediate protein-protein interactions, we can imagine that interactions between ISG20 and other proteins are required to mimic, in human, the XPMC2 function. However, we have no direct evidence to accredit this hypothesis at present. In addition, ISG20 shares a strong amino acid homology with a theoretical ORF present in the genome of *S. cerevisiae*. The function of this ORF remains unknown. Since protein conservation during evolution usually affects essential cellular functions, the comprehension of the regulation and biological activity of the *S. cerevisiae* ORF will be important to determine the actual role of ISG20. The knock-out of the yeast gene is now in progress to allow this study.

We have examined the subcellular localization of ISG20 protein. Ectopic expression of a fusion protein between ISG20 and the HA epitope peptide revealed that ISG20 is predominantly nuclear and gives a punctuate staining pattern. Interestingly, the number and size of the nuclear dots containing ISG20 were variable from one cell to another. These data can reflect a modulation of PML NBs throughout the cell cycle. Using confocal immunofluorescence microscopy, we demonstrated that ISG20 is closely associated with PML and SP100 within the large multiprotein complexes termed PML NBs (19–28). To determine whether a direct interaction between these proteins can occur, a yeast two-hybrid study was performed. This was conducted using ISG20 fused to the GAL4 DNA-binding domain and PML or SP100 fused to the GAL4-activating domain. No direct interaction was detected between these proteins when coexpressed in yeast Y187 cells.

Viruses require the host cell machinery for their multiplication cycle, and they have developed various strategies to circumvent the antiviral activities induced in the cells by IFNs. Various viral proteins such as the adenovirus E4-ORF3 protein (37, 38), the human T-cell leukemia virus type 1 Tax oncoprotein (39), the herpes simplex virus type 1 Vmw110 protein (40, 41), the cytomegalovirus promoter IE1 protein (43), and the SV40 large T antigen (37) concentrate in the PML NB speckles, suggesting that PML NBs play a major role during infection by oncogenic viruses. The fact that the PML NB-associated proteins PML, SP100, NDP52, and ISG20 are all inducible by IFNs (Refs. 19–22 and this report) strongly suggests the implication of a such subnuclear structure in the mechanism of

IFN action. Further studies on the modulation of Int-6 and PIC-1, a newly described PML NB-associated protein (58, 59), by IFNs might be worth being performed to confirm this hypothesis. The disruption of PML NBs during viral infection reflects the ability of viruses to circumvent the antiviral activities of IFNs. Eventual modifications of ISG20 subcellular localization after various viral infections are underway to increase the understanding of the role of ISG20 in the mechanism of antiviral action induced by IFNs. The availability of specific antibodies against this protein is essential to determine its function, and their preparation is now in progress.

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